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PROCEEDINGS

Mechanisms of Reproducible Microbial Pitting of 304 Stainless Steel by a Mixed Consortium Containing Sulphate-Reducing Bacteria.

Dr. Peter Angell*

Dr. Jiunn-Shyong Luo

Dr. David C. White

Center for Environmental Biotechnology

10515 Research Drive, Suite 300

Knoxville, TN. 37932-2575

Abstract

Pitting corrosion is characterised by the formation of small anodes and corresponding large cathodes, which allow the formation of pits in the metal matrix. A number of mechanisms have been put forward to account for the involvement of SRB in this process including: cathodic depolarization of hydrogen, bacterial sulphide acting as the cathode, and changes in pH due to sulphate-reducing bacteria (SRB). To date there has been little success in reproducibly inducing SRB pitting of stainless steels in a laboratory system.

A concentric electrode has been designed in which a small anode is artificially produced within a large cathode by applying a galvanic current of 12 μA cm^{-2}. During the application of this galvanic current, various consortia and axenic cultures of bacteria are allowed to colonise the surface of the stainless steel concentric electrode. After seventy-two hours the induced current is removed and the resultant current monitored. Electrochemical impedance spectroscopy (EIS) is carried out on both the anodes and cathodes in order to determine the charge transfer resistance (Rct values) and changes in phase angles with time. At the termination of the experiment viable counts of both SRB & *Vibrio* sp. are recorded from the anodes and cathodes.

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A coupling current of about 6 μ A cm^{-2} is maintained only in the presence of a mixed culture containing an SRB and a *Vibrio* sp.. In order for the current to be maintained, a low Rct value is necessary at the anode <3 KΩ cm^{-2}. It appears that the mixed culture including SRB is necessary to maintain this low Rct value. Also, the cathode must have a high Rct value in excess of 100 KΩ cm^{-2}. This high value would appear to be dependant on the presence of the SRB on the cathode. It is also tentatively suggested that the magnitude of the resultant coupling current is a function of the number of bacteria on the anode.

These results indicate that microbial corrosion is not a simple process involving one bacteria and one mechanism. Rather, it is a complex phenomenon with mixed consortia working through a number of mechanisms.

Introduction

The microbial involvement in the corrosion (MIC) of steels has been recognized for many years, with a great deal of research having been carried out as reviewed by several authors [1,2]. The bulk of the literature to date deals with the effects of the sulphate-reducing bacteria (SRB) on mild steel. This has been due in part to two main factors. First, a vast quantity of mild steel is used in pipe lines, in marine and freshwater systems, where MIC has been seen to be a significant problem [3]. Second, mild steel is notably less resistant to microbial attack than stainless steels. The latter have proved to be notoriously difficult to obtain reproducible pitting in the laboratory. Most of the evidence that SRB are involved in MIC of stainless steel has come from their isolation form failures in field systems. The presence of sulphide in the regions of the pits has also served to further implicate the SRB in the process

The heat treated zone of weldments are the areas in which most SRB failures of stainless steels have been reported to have occurred. The pits are characterized as being steep sided and often form a network of tunnels within the metals, reminiscent of Swiss cheeses. The resistance of stainless steel is thought to be due to the production of a passive film on the metal surface. The location of the pits, normally in the heat treated zone is also of significance as in this region the crystal lattice and surface chemistry have been altered by the elevated temperatures encountered during the welding process.

The vast majority, if not all, the literature on MIC of steels to date

has been concerned with either the reporting of field failures or test systems set up in natural flowing waters. To the knowledge of these authors no system has been reported to date in which reproducible pitting has been described in a laboratory system relating to the action of SRB on stainless steel. Field data has shown that stainless steels exposed to marine environments undergo ennoblement in which the open circuit potential (OCP) rises to the critical pitting range [4]. This ennoblement can be linked to the formation of a biofilm on the surface acting as an oxygen barrier decreasing the dissolved oxygen tension at the surface of the metal.

To date the primary mechanisms advanced for the MIC of steels by SRB have involved cathodic reactions as reviewed by Hamilton [5]. One mechanism involved the depolarization of the cathode by the reduction of cathodic hydrogen by the enzyme hydrogenase. The other involved bacterially produced iron sulphide acting as a cathode. Recently evidence has been suggested that a microbial biofilm is capable of creating corrosive metabolites such as hydrogen peroxide[6]. Also very low pHs, below 2, have been detected below naturally forming biofilms on stainless steel exposed to water in the Chesapeake Bay area[6],

In this paper a laboratory system will be described which has could reproducibly sustain a corrosion current between an artificially generated anode and cathode in the presence of SRB. This system has facilitated the study of the mechanisms, and role of SRB in the MIC of stainless steel leading to some new insights into the action of these bacteria. The system is also suited to further studies concentrating on the mechanisms of pitting. These studies will allow for the determination of whether a link between bacterial metabolism and corrosion exists. Also, studies on the effect of the elevated temperatures encountered during welding are possible.

Materials and Methods

Bacteria used were a culture of *Desulfovibrio vulgaris* and an unidentified *Vibrio* sp.. The *Vibrio* was used due to its being a facultative anaerobe. Also, *Vibrio natrigens* has been shown by some reports to be involved in the MIC of metals[7]. The SRB was also found to tolerate low levels of oxygen. No quantification was carried out on the level of oxygen tolerance, but it was not killed by exposure to air during the plating procedure described below. It was also found to grow in previously degassed MPN tubes which had been opened and left open to the atmosphere in a laminar flow hood.

Concentric electrodes were machined from 304 stainless steel rod providing a small anode with a large surrounding cathode. The anode had a surface area of 0.031 cm^{-2} and the cathode had a surface area of 4.87 cm^{-2}. The anode was electrically isolated from the cathode by a Teflon spacer, care was taken in the machining of each component of the concentric electrode to ensure a snug fit. Electrical wire was soldered to the back of each electrode before the whole assemble was mounted in a cold cure epoxy. The electrical wire was passed through 1.5 cm length of 16 gauge silicone tubing. Half of the silicone tubing was sealed into the epoxy the other half was used to secure a piece of glass tubing to allowing positioning of the electrode within the reaction vessel. The electrical wires were passed through the center of the tubing to the outside of the vessel. The mounted concentric electrode was polished to a 600 grit finish using an abrasive paper. The polished electrode was then degreased by sonicating in acetone before being air dried.

The reaction vessel comprised a 600 ml glass vessel with a 316 stainless steel top. A number of ports were provided in the top sealed with compression fittings, allowing for: medium addition and removal, gas sparging and venting, a circulation loop to aid mixing in the vessel and ports for a titanium counter electrode and caramel reference electrode. The reference electrode was electrolytically connected to the vessel via a Lugin's bridge. The medium feed contained a drip tube to prevent bacterial grow back, of the bacteria, to the medium reservoir and cross contamination of the vessels.

For the initial part of the experiment during bacterial inoculation the system was run as a batch reactor to enhance bacterial attachment to the concentric electrode. The reactor vessel was sparged with a 95 % nitrogen 5 % hydrogen gas mixture to ensure anaerobic conditions. During this period mixing was carried out using a magnetic stirrer operating at approximately 100 rpm. Following the batch phase flow was initiated at 1 ml min^{-1} giving a dilution rate of 0.1 h^{-1}. The systems were operated at room temperature (25 °C ± 2 °C). As electrochemical measurements were being made on the flowing system the magnetic stirrers were replaced by a circulation loop in which medium was pumped from the bottom of the vessel to the top.

In order to initiate the pitting for the first seventy-two hours while the bacteria were cultured under static conditions a galvanic current was applied to the concentric electrode. The current was applied via a

galvanostat such that an anodic current density of 12 $\mu A\ cm^{-2}$ was maintained. At the end of this period of time the current was removed and the resultant current flowing between the anode and cathode was monitored. At various time points throughout the flowing cycle of the experiment electrochemical impedance measurements were made in order to determine the charge transfer resistance (Rct) of each of the anodes and cathodes. The Rct value is the inverse of the I cor the corrosion current, a measure of the rate of corrosion.

At the end of the experiment the concentric electrodes were removed from the vessels and the numbers of bacteria on each of the anodes and cathodes were enumerated. Enumeration was carried out by both the MPN method and plate counts on Iverson's Medium [8] using the technique of Miles and Misra [9]. Replicate plates were incubated both aerobically and anaerobically to provide counts on both SRB and the facultative *Vibrio* sp..

Results and discussion

Figure 1 shows a plot of the current density at the anode as measured over one hundred and twenty hours. It shows that in the presence of a mixed culture after the applied current is removed, after 72 hours, a current density of approximately 3 $\mu A\ cm^{-2}$ was maintained for the remainder of the experiment. However, this was not the case with either the axenic SRB, axenic *Vibrio* or the sterile control. This current is the actual corrosion current flowing between the anode and cathode and is therefore an accurate measure of the rate of corrosion.

The Rct values indicated that the lowest value is seen for the anode ($< 1\ K\Omega \cdot cm^2$) of the mixed culture in which corrosion was taking place. This system also showed the highest value for the cathode ($> 100\ K\Omega \cdot cm^2$). No other system showed such low anodic values, although similar high values were seen for the cathode in the SRB systems. All the other values fell between the two and were normally in the range of 10 $K\Omega \cdot cm^2$ to 80 $K\Omega \cdot cm^2$.

Table 1 shows the numbers of bacteria isolated from the various electrodes exposed to different conditions as well as the level of any current maintained. From this table it is seen that both SRB and the *Vibrio* were necessary on both the anode and cathode. In one experiment were a low number of SRB (10^4 cfu cm^{-2}) were present on the anode showed that the current was not maintained but slowly

dropped to zero over approximately twenty-four hours, as opposed to the sharp drop seen with the axenic cultures and the sterile control.

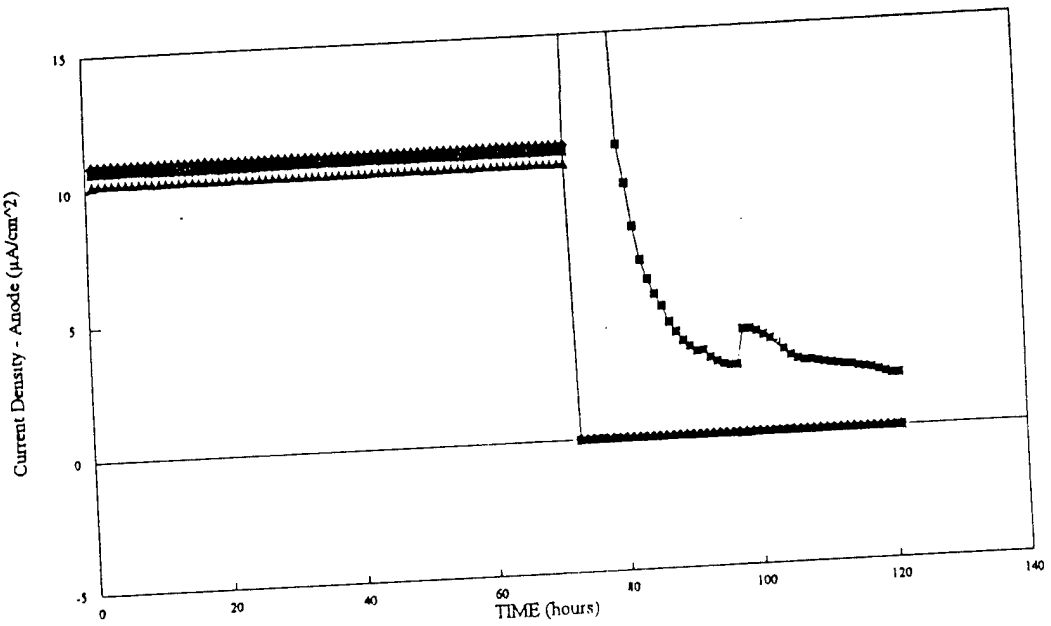

Figure 1 Time plot of measured current flowing between the anode and cathode; ■ mixed culture of *Desulfovibrio vulgaris* and *Vibrio* sp with a current maintained after applied current is removed, other plots are axenic SRB, axenic *Vibrio* and sterile showing no maintainned current.

Table 1. Bacterial numbers and coupling current density for concentric stainless steel coupons with various treatments.

Coupon Treatment	SRB (cfu/ml)		*Vibrio* (cfu/ml)		Coupling Current
	Anode	Cathode	Anode	Cathode	
Sterile	-	-	-	-	-
Axenic SRB	3.2×10^4	2.0×10^1	-	-	-
Axenic *Vibrio*	-	-	1.0×10^8	1.0×10^7	-
Mixed	1.0×10^7	1.0×10^6	1.0×10^8	1.0×10^6	3 µA cm^{-2}
Mixed	3.2×10^4	2.0×10^5	1.6×10^7	2.8×10^5	*

* slow decline to zero

Figure 2. shows a run in which the corrosion current was maintained in the presence of a mixed culture for 30 hours at which point 10 mM sodium azide was added to the culture. Sodium azide is an inhibitor of aerobic respiration blocking the transfer of electrons from cytochrome a and a1 to O_2. This system is not operative in the SRB although reports have suggested that sodium azide inhibited the growth of *Desulfovibrio* while actually stimulating the rate of sulphate reduction in H_2 [10]. On the addition of the sodium azide it can be seen that there was no effect of the level of the current density at the anode, there was no inhibition or enhancement.

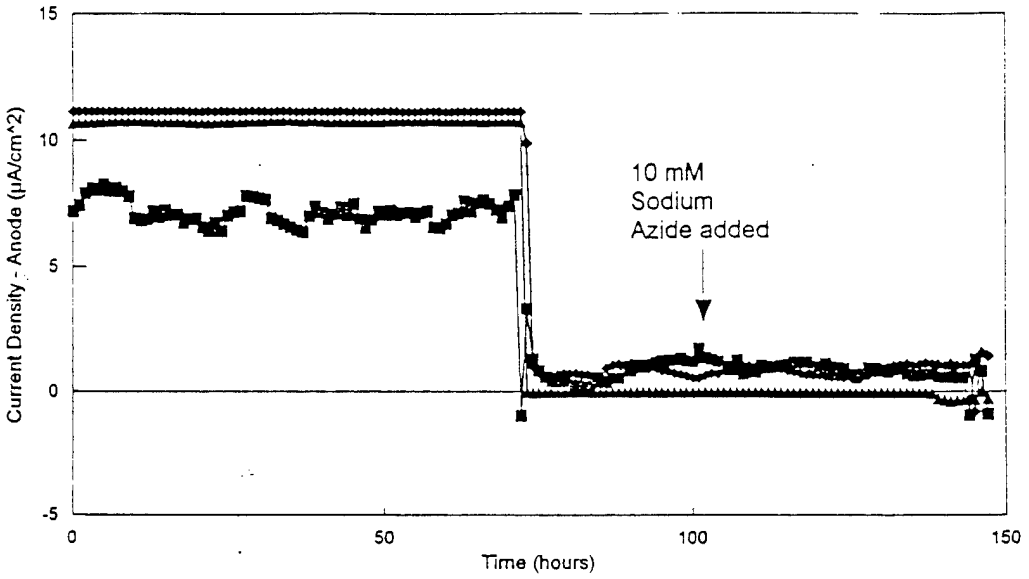

Figure 2 Time plots of measured current flowing between anode and cathode for: (■) & (♦) mixed culture of *Desulfovibrio vulgaris* and *Vibrio* sp, (▲) sterile control, with the addition of 10 mM sodium azide at 100 hours, note sodium azide is an inhibitor of aerobic respiration blocking the transfer of electrons from cytochrome a and a1 to O_2 and has no effect on the current being maintained by the mixed culture.

SRB use sulphate as their terminal electron accepter instead of oxygen in which the sulphate is reduced to sulphide. One of the theories for the mechanism for SRB pitting is that this sulphide production acts as a cathode. If the sulphate is removed form the bacteria they are unable to produce the sulphide which if the theory is correct will stop the corrosion. In order to test this hypothesis various experiments were tried initially growing the bacteria in the absence of sulphate while the current

was being applied and then adding sulphate to the medium when the applied current was removed (Figure 2.). In this case there was no maintenance of the corrosion current even though both the *Vibrio* and the SRB were isolated from both the anode and cathode at the termination of the experiment. Similar results were seen when the experiment was reversed and the bacteria were grown in the presence of sulphate and then it was removed when the applied current was removed.

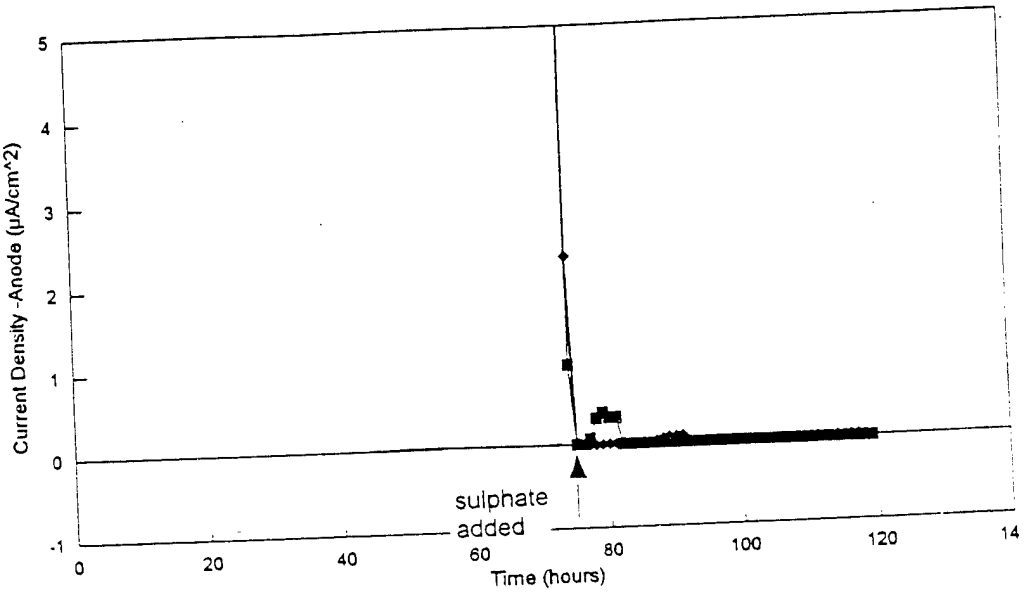

Figure 3 Time plot of measured current flowing between anode and cathode for mixed culture of *Desulfovibrio vulgaris* and *Vibrio* sp in the absence of sulphide while the current was applied and addition of sulphate to the medium when the applied current was removed, showing that no current was maintained.

Figure 4. shows a plot of current density with time for a run in which the electrodes were colonized with mixed bacteria in the presence of sulphate for 4 days. The medium was then drained and the vessels rinsed with sulphate free medium which was used through out the period that the current was applied and the system was static. When the applied current was removed sulphated medium was again added as an open system was operated. In this case the current was maintained. After it had been maintained for fifty hours the medium was again changed to one that contained no sulphate. This was fed to the vessel for a further 60 hours during which time 10 volumes had been replaced,

which should have been sufficient to remove any residual sulphate. However, this had no effect on the level of the coupling current. In fact the current was maintained for a further forty hours during which time no fresh medium was added.

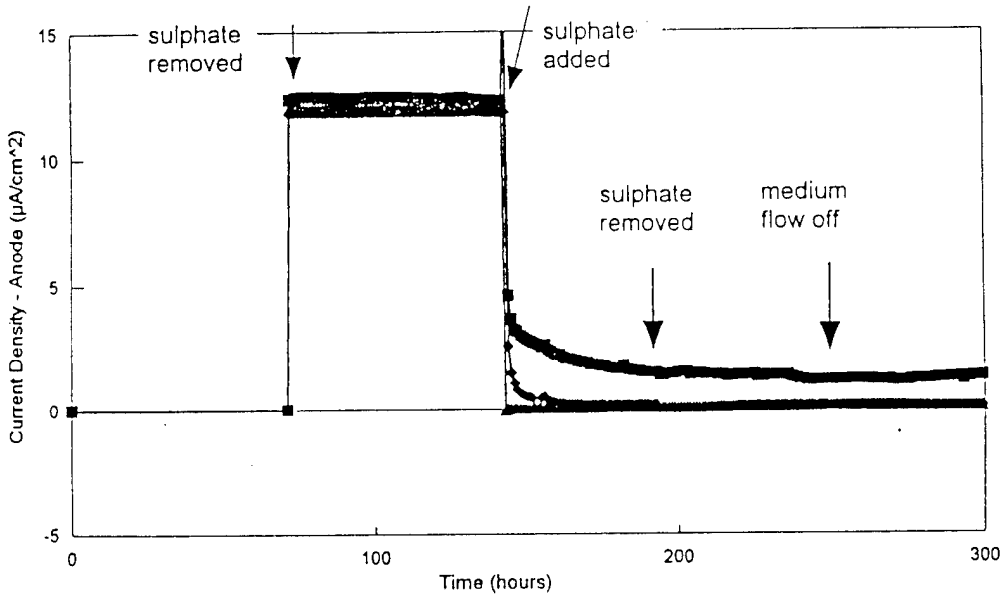

Figure 4 Time plot of measured current flowing between the anode and cathode for (■) and (♦) mixed culture of *Desulfovibrio vulgaris* and *Vibrio* sp. (▲) sterile control where sulphate was added for an initial colonisation period of 72 hours while no current was applied, sulphate was then removed while the current was applied and added again when the applied current was removed later sulphate was removed and experiment continued after medium flow was stopped. The current was maintained in one of the mixed cultures even after sulphate was removed and the medium flow was stopped.

Figure 5. shows a similar run in which sulphate was only added while the electrodes were being colonized. After this point for the rest of the experiment sulphate free medium was used. In this case only a very low level of current ($<$o.1 μA cm^{-2}) was maintained. This level of current is higher than that seen in the sterile control but is far lower than those seen previously when sulphate was present.

It should be noted that the removal of the sulphate could be having one of two effects either it prevented the production of sulphide but allowed the cells to continue metabolism or it prevented metabolism and sulphide production. Certain *Desulfovibrio* sp. have been shown to be able to grow in the absence of sulphate using nitrate instead as the

terminal electron acceptor[11]. Preliminary studies using culture tubes suggest that the growth of the *Desulfovibrio vulgaris* strain used in this experiment was capable of very limited growth in the absence of sulphate at best. Further studies are planned utilizing the incorporation of radio-labelled acetate into the lipids of the bacteria as an indicator of growth in the absence of sulphate. Also stains will be examined and tested which have been reported to reduce nitrate in the absence of sulphate.

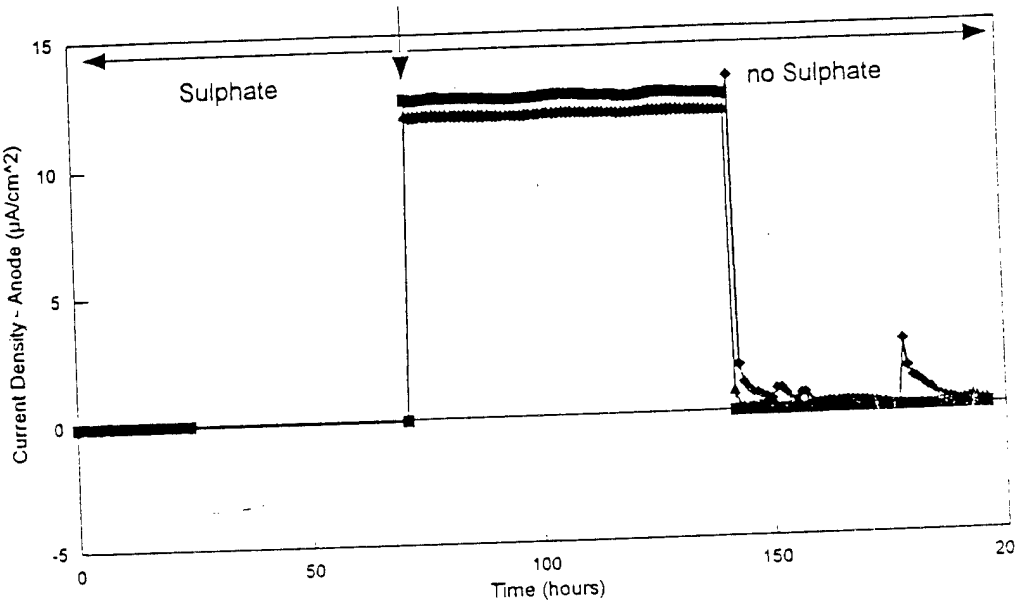

Figure 5 Time plot of current flowing between anode and cathode for (◆) and (▲) mixed culture of *Desulfovibrio vulgaris* and *Vibrio* sp., (■) sterile control where sulphate was added for the initial colonistaion period but not for the remainder of the experiment showing that very little current was maintained in the mixed cultures.

From these results it would appear that sulphate is not necessary during the conditioning phase for the maintenance of the current. As noted above it is likely that the sulphate has a double effect of not only stopping the production of sulphide but also markedly reducing the metabolism of the cells. It is therefore not possible to determine to which effect the inability to maintain the current should be ascribed. However, it does appear that corrosion can be maintained when metabolism/sulphide production is only inhibited during the initiation phase and not subsequently. It would also appear that once the corrosion is initiated in the presence of sulphate metabolism/sulphide

production is no longer necessary. This could be attributed to the initiation of an electrochemical cell by the bacteria, that reaches a stage where it becomes self sustaining.

Further work is planned using a total metabolic inhibitor such as 2-heptyl-4-hydroxyquinoline N-oxide which blocks the electron transport chain between cytochrome b and cytochrome c which should be present in the SRB. It is also noted that few *Desulfovibrio* sp utilise nitrate in place of sulphate but the majority are able to use nitrite instead[12]. This will be tested in the SRB used in this study.

Acknowledments

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